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High-performance liquid chromatography method for the quantification of rabeprazole in human plasma using solid-phase extraction

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Abstract

A simple, sensitive and selective HPLC method with UV detection (284 nm) was developed and validated for quantitation of rabeprazole in human plasma, the newest addition to the group of proton-pump inhibitors. Following solid-phase extraction using Waters Oasis™ SPE cartridges, the analyte and internal standard (Pantoprazole) were separated using an isocratic mobile phase of 5 mM ammonium acetate buffer (pH adjusted to 7.4 with sodium hydroxide solution)/acetonitrile/methanol (45/20/35, v/v) on reverse phase Waters symmetry® C_{18} column. The lower limit of quantitation was 20 ng/mL, with a relative standard deviation of less than 8%. A linear range of 20–1000 ng/mL was established. This HPLC method was validated with between- and within-batch precision of 2.4–7.2% and 2.2–7.3%, respectively. The between- and within-batch bias was −1.7 to 2.6% and −2.6 to 2.1%, respectively. Frequently coadministered drugs did not interfere with the described methodology. Stability of rabeprazole in plasma was excellent, with no evidence of degradation during sample processing (autosampler) and 3 months storage in a freezer. This validated method is sensitive, simple and repeatable enough to be used in pharmacokinetic studies.

Keyword: Rabeprazole

1. Introduction

Rabeprazole, 2-[[[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]-methyl]sulfinyl]-1H-benzimidazole (Fig. 1), is a newly developed benzimidazole proton-pump inhibitor, which suppress gastric acid secretion by specific inhibition of the H^+/K^+-ATPase enzyme system at the secretary surface of the gastric parietal cell [1]. Rabeprazole is used for the treatment of acid-peptic diseases, such as duodenal, gastric and oesophageal ulceration [2]. Proton-pump inhibitors (e.g. omeprazole, lansoprazole, pantoprazole and rabeprazole) are activated by conversion to sulphenamides in the acid environment. Activation of rabeprazole is significantly faster, in line with its more unstable chemical structure [3]. In line with the inherent instability of the parent molecule, rabeprazole is also less dependent than the other drugs on pH for conversion to the chemically active sulphenamide form [3]. Therefore, establishing a reliable method for the quantification of rabeprazole in plasma, its stability must be considered during sample collection and handling.

A survey of the literature revealed that there have been very few methods for rabeprazole quantification in plasma [4–6]. Nakai et al. [4] established a method for the simultaneous determination of rabeprazole and its four metabolites in human plasma by HPLC with UV absorbance detection. The quantification limit was 5 ng/mL for rabeprazole and 20 ng/mL for each of its four metabolites using a 1 mL of plasma sample, but the sample preparation was complex with double extraction. They investigated the stability of rabeprazole in human plasma after short- and long-term storage, but the stability after sample preparation and during the analytical process was not reported. Takakuwa et al. [5] and Mano et al. [6] reported a gradient HPLC method for quantification of rabeprazole in plasma that required a long run time (>25 min)

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and the LLOQ (30 ng/mL) was high, where the stability of rabeprazole was not reported. El-Gindy et al. [7] used spectrophotometric and chromatographic methods to study the stability of rabeprazole towards acidic, oxidative and photo degradation processes. Further, they investigated the kinetics of the oxidative and photo degradation processes, and calculated the activation energy for rabeprazole degradation. It was found that rabeprazole was rapidly degraded in acid medium and was more stable in alkaline. Radi et al. [8] investigated voltammetric behavior of rabeprazole at a glassy carbon electrode in Britton-Robinson buffer solutions. Recently, Zhang et al. [9] reported a liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method in selected reaction monitoring mode using an atmospheric pressure chemical ionization source for the quantification of rabeprazole in human plasma using omeprazole as the internal standard in the range 2–800 ng/mL. Although these methods meet many or all generally accepted criteria for validated analysis of rabeprazole, they are often cumbersome, have a very long sample preparation and/or chromatographic time or involve costly equipment (LC–MS/MS).

The objective of the present work is to establish a fully validated HPLC method with a quantitation limit sufficiently low to support pharmacokinetic and bioequivalence of rabeprazole single/multiple dose studies. The described method provides information about the stability of rabeprazole both in plasma and during sample processing (autosampler), which is a clear advantage for determining a large number of plasma samples for pharmacokinetic and bioequivalence studies in patients and healthy subjects.

2. Experimental

2.1. Chemicals

Rabeprazole sodium and pantoprazole (internal standard, I.S.) drug substances were obtained from Cadila Healthcare Limited (Ahmedabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were from Merck (Darmstadt, Germany). Ammonium acetate, diethylamine and sodium hydroxide were purchased from Merck (Worli, Mumbai, India). Oasis HLB extraction cartridges (30 mg, 1 mL) from Waters, USA. HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. Chromatography

The integrated high performance liquid chromatography system (LC 2010C, Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary pump, a degasser, an autosampler, an injector with a 100-μL loop, a column oven, a UV detector and a data system (Class VP version 6.12). The separation of compounds was made on a Waters symmetry®

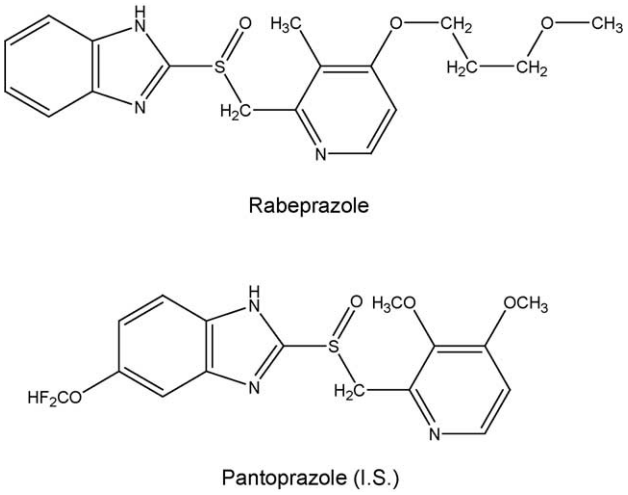

Fig. 1. Chemical structures of rabeprazole and pantoprazole.

C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) at 30 °C temperature. The mobile phase was a mixture of 5 mM ammonium acetate buffer (pH adjusted to 7.4 with sodium hydroxide solution)/acetonitrile/methanol (45/20/35, v/v) pumped at a flow-rate of 1.0 mL/min. Detection was set at a wavelength of 284 nm.

2.3. Solid phase extraction

Plasma sample (1 mL) was pipetted into microcentrifuge tubes and spiked with 50 μL of I.S. working solution (20 μg/mL). The mixture was vortex mixed for 10 s and loaded onto an OASIS HLB SPE cartridge (C_{18}, 30 mg, Waters Co., Milford, USA), which has been conditioned by washing with methanol (1 mL) followed by water (1 mL). The OASIS cartridge was drained with a vacuum manifold system, washed with 5 mM ammonium acetate, pH 10.0 (2 mL) followed by 20% methanolic solution of 5 mM ammonium acetate, pH 10.0 (1 mL) and then eluted with methanol (2 mL). The eluate was completely evaporated to dryness using a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Then, the dried extract was reconstituted in 150 μL of diluent (5 mM acetate buffer, pH adjusted to 10 with sodium hydroxide solution) and a 100-μL aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Standard stock solution of rabeprazole (1 mg/mL) was prepared in 0.1% methanolic solution of diethylamine. Standard stock solution of I.S. (1 mg/mL) was prepared in acetonitrile. These solutions are protected from light by covering them with aluminium foil. Working solutions were prepared by appropriate dilution in diluent just before use, using amber glass volumetric flasks in order to avoid photo degradation.

All solutions were stored in darkness at 4 °C. The I.S. working solution (20 μg/mL) was prepared by diluting stock solution with water–acetonitrile (50:50, v/v). Fifty microliters of working solutions were added to 950 μL of drug-free plasma to obtain rabeprazole concentrations of 20, 30, 50, 100, 200, 300, 500 and 1000 ng/mL. The quality control samples were prepared in pool, at concentrations of 20 ng/mL (LLOQ), 60 ng/mL (low), 600 ng/mL (medium) and 800 ng/mL (high), as a single batch at each concentration, and then divided in aliquots that were stored in the freezer at below −50 °C until analysis.

A validation run consists of double control, system suitability sample, a blank sample (a plasma sample processed without an I.S.), a zero sample (a plasma processed with I.S.), and a calibration curve with eight non-zero samples covering the total range (20–1000 ng/mL) and QC samples at three concentrations ($n = 6$, at each concentration). Such validation runs were generated on 5 consecutive days. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

2.4.2. Specificity

Randomly selected blank human plasma samples, which were collected under controlled conditions, were carried through the extraction procedure and chromatographed to determine the extent to which endogenous plasma components may contribute to interference with the analyte or the internal standard. The results were compared with the results to those obtained from a solution with a drug concentration nominally at 20 ng/mL of an extracted rabeprazole plasma sample.

2.4.3. Recovery

Recovery of rabeprazole was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six neat reference solutions (unprocessed). Recovery of pantoprazole (I.S.) was evaluated by comparing the mean peak areas of 10 extracted quality control samples to mean peak areas of 10 neat reference solutions (unprocessed) of the same concentration.

2.4.4. Accuracy and precision

Within-batch accuracy and precision evaluations were performed by repeated analysis of rabeprazole in human plasma. The run consisted of a calibration curve plus six replicates of each LLOQ, low, medium and high quality control samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and six replicates of LLOQ, low, medium and high quality control samples for rabeprazole on three separate occasions.

The overall precision of the method expressed as relative standard deviation and accuracy of the method expressed in terms of bias (% deviation from true value).

2.4.5. Stability

The short-term stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability of rabeprazole was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 30 h. Stability of rabeprazole in human plasma was tested after storage at below −50 °C for 3 months. The stability of standard solutions was also tested at room temperature for 2, 24 h and upon refrigeration (4 °C) for 14 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of rabeprazole after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

3. Results and discussion

3.1. Sample preparation

Solid-phase extraction using OASIS HLB SPE cartridges was chosen for a fast and easy sample preparation. It was reported that a buffer of pH 10.4 was added to plasma samples before liquid–liquid extraction [4,5], which made rabeprazole changed into sodium salt and avoided acidic degradation [7], but it needed twice extraction with ethylacetate. Therefore, 5 mM ammonium acetate buffer pH 10 and 20% methanolic solution of 5 mM ammonium acetate buffer pH 10 were employed in this extraction procedure for washing and to avoid acidic degradation. Even the dried extracts were reconstituted in 5 mM ammonium acetate buffer pH 10 to avoid acidic degradation.

3.2. Separation

To achieve better separation and good peak shapes, the mobile phase containing varying percentages of organic phase and pH modifiers were tested. At the same time, the stability of rabeprazole in the mobile phase was also examined. Using mobile phase of water–methanol (20:80, v/v), rabeprazole degraded more than 20% within 10 min. The stability of rabeprazole is improved using the mobile phase containing 5 mM ammonium acetate buffer pH 7.4. Therefore, mixture of 5 mM ammonium acetate buffer (pH adjusted to 7.4 with sodium hydroxide solution)/acetonitrile/methanol (45/20/35, v/v) was used as the mobile phase, which shortened analytical time (<10 min) and avoided the degradation of rabeprazole. The analyte and I.S. were well separated from co-extracted material under the described chromatographic conditions at retention times of 7.6 and 6.6 min, respectively. The peaks were of good shape, completely resolved one from another at therapeutic concentrations of rabeprazole. No interference

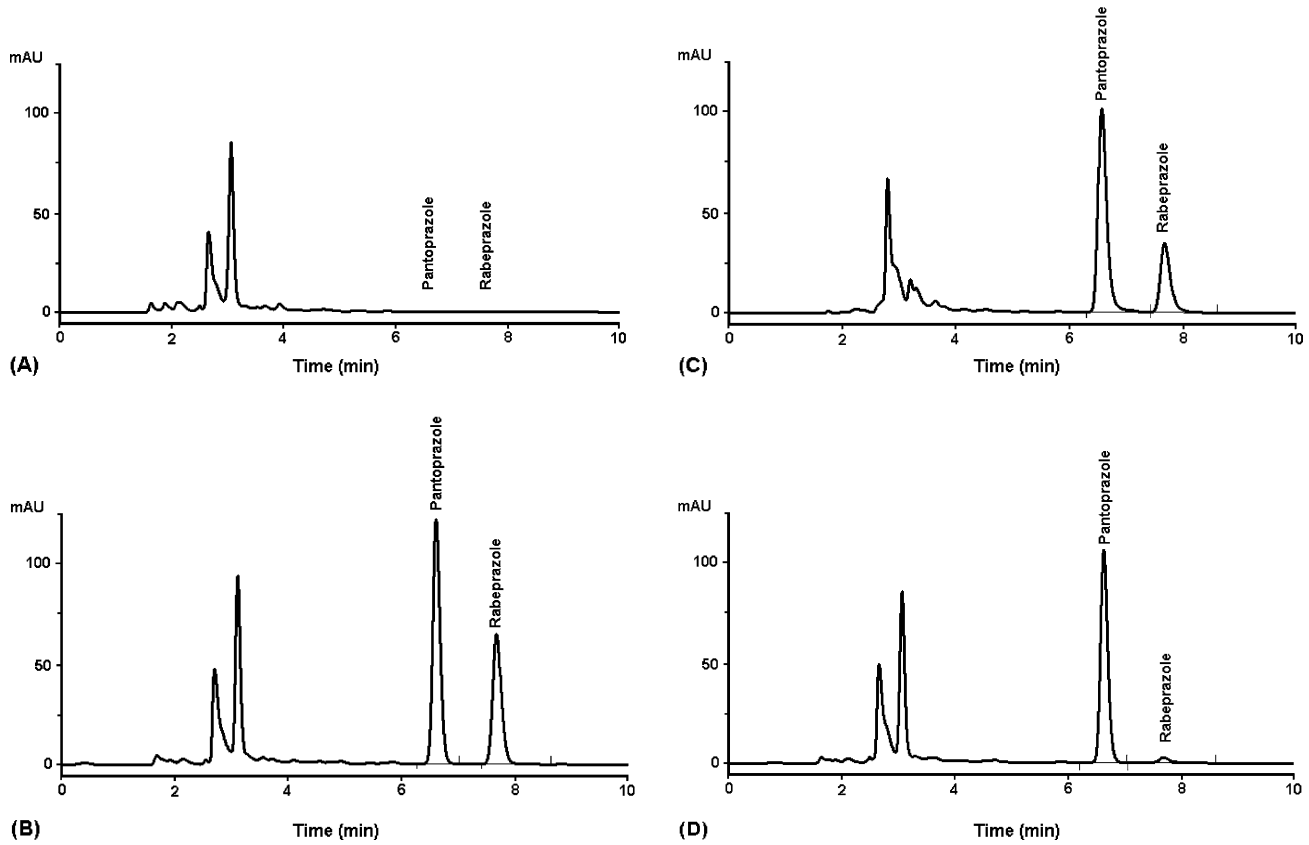

Fig. 2. Chromatograms of (A) blank human plasma; (B) human plasma sample spiked with 600 ng/mL of rabeprazole and I.S.; (C) plasma sample from a healthy subject following a 20 mg oral dose of rabeprazole, the plasma concentration was determined to be 311.96 ng/mL for rabeprazole; (D) spiked human plasma sample at LLOQ (20 ng/mL). Approximate retention times: rabeprazole = 7.6 min; I.S. = 6.6 min.

with constituents from the plasma matrix was observed. Fig. 2 shows the representative chromatograms of blank plasma, plasma samples spiked with rabeprazole at 600 ng/mL and at LLOQ (20 ng/mL), and plasma sample obtained from a healthy subject following an oral 20 mg dose of rabeprazole.

3.3. Linearity and sensitivity of the assay

Linear least-square regression analysis of the calibration graph on 5 different days demonstrated, linearity between the response and the nominal concentration of rabeprazole over the range of 20–1000 ng/mL. The lower limit of quantitation (LLOQ) was 20 ng/mL. This LLOQ is sufficient to support rabeprazole bioequivalence studies. The mean linear regression equation of calibration curve for the analyte was $y = 643.37(\pm 151.14)x + 3.61(\pm 1.09)$, where y was the peak area ratio of the analyte to the I.S. and x was the concentration of the analyte. The correlation coefficient (r) for rabeprazole was above 0.99 over the concentration range used. Table 1 summarizes the calibration curve results for the analyte. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between- and within-batch validations.

Table 1
Statistical evaluation of the analysis results for rabeprazole in standard curves during 5 days

Concentration added (ng/mL)	Concentration found (mean ± S.D., $n = 5$) (ng/mL)	Precision (%)	Bias (%)
20	20.3 ± 0.3	1.6	1.9
30	30.1 ± 1.5	5.0	0.2
50	49.5 ± 3.1	6.3	−0.9
100	97.5 ± 5.4	5.5	−2.4
200	199.7 ± 12.9	6.5	−0.1
300	289.2 ± 14.1	4.8	−3.5
500	509.3 ± 12.4	2.4	1.8
1000	1039.4 ± 48.1	4.6	3.9

3.4. Extraction

The extraction recovery of rabeprazole was 84.5% on average, and the dependence on concentration is negligible. The recovery of I.S. was 43.1% at the concentration used in the assay (20 μg/mL). Recovery of I.S. was low, but it was consistent, precise and reproducible.

3.5. Specificity

There were no interfering peaks present in six different randomly selected samples of drug free human plasma used

Table 2
Accuracy and precision of the HPLC method for determining rabeprazole concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision ($n = 6$)			Between-batch precision ($n = 3$)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Bias (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Bias (%)
20	20.4 ± 1.1	5.3	2.1	20.5 ± 1.4	7.2	2.6
60	58.4 ± 4.2	7.3	−2.6	58.9 ± 3.1	5.3	−1.7
600	592.2 ± 17.2	2.9	−1.2	600.2 ± 14.6	2.4	0.1
800	782.2 ± 17.6	2.2	−2.2	801.1 ± 22.1	2.7	0.1

for analysis at the retention times of either analyte or internal standard. There was no interference of rabeprazole and pantoprazole analysis by other potentially co-administered drugs such as paracetamol, nicotinamide, ibuprofen, caffeine, aspirin, omeprazole, lansoprazole, ampicillin, amoxicillin, loratadine, desloratadine, atorvastatin, clopidogrel, metformin, glimepiride, celecoxib, rofecoxib, valdecoxib, etoricoxib, naproxen and nimuselide.

3.6. Accuracy of the assay

The accuracy values for between- and within-batch studies at the LLOQ and at low, medium and high concentrations of rabeprazole in plasma were within acceptable limits ($n = 3$) (Table 2).

3.7. Precision of the methods

3.7.1. Within-batch variability of the assay

The results shown in Table 2 indicates that the assay method is reproducible for replicate analysis of rabeprazole in human plasma within the same day.

3.7.2. Between-batch variability of the assay

The results shown in Table 2 indicate sthat the assay method is reproducible on different days.

3.8. Stability

The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. These were performed as described in Section 2.4.5. Acceptable analyte stability was demonstrated for all phases of storage and processing. All stability results are summarized in Table 3. Three freeze–thaw cycles and 24 h room temperature storage for low and high quality controls samples indicated that rabeprazole was stable in human plasma under these conditions. QC samples were stable for at least 3 months if stored frozen at below −50 °C. These stability results were similar to those reported in the literature [5]. In addition, the stability of processed plasma sample was also tested. It was found that rabeprazole was stable at room temperature for 4 h and at 4 °C (autosampler) for 30 h after the reconstitution of residue of plasma extract with 5 mM ammonium acetate buffer (pH 10), but it degraded more than 60% at room temperature after 30 h, suggesting that plasma samples after extraction should be analyzed within 4 h if left at room temperature or within 30 h if left at 4 °C. The standard solutions of rabeprazole in 0.1% methanolic solution of diethylamine allowed to stand at room temperature 2, 24 and at 4 °C for 14 days. The coefficient of variation for each QC levels was within 0.3, 7.1 and 7.5%, respectively. I.S. stock solution was prepared in 0.1% methanolic solution of diethylamine and its working solution (20 μg/mL) by diluting the stock solution with diluent (5 mM acetate buffer, pH

Table 3
Stability of the samples

Sample concentration (ng/mL)	Concentration found (ng/mL)	Precision (%)	Bias (%)
Short term stability for 24 h ($n = 6$) in plasma			
60	62.7	5.5	4.5
800	794.5	6.7	−0.7
Three freeze and thaw cycles ($n = 6$)			
60	57.5	6.7	−4.2
800	825.5	9.8	3.2
Autosampler stability for 30 h ($n = 6$) (after extracting and reconstitution)			
60	55.5	4.4	−7.5
800	735.9	6.6	−8.0
3 months stability at <−50 °C ($n = 6$)			
60	54.1	8.9	−9.8
800	724.9	13.4	−9.3

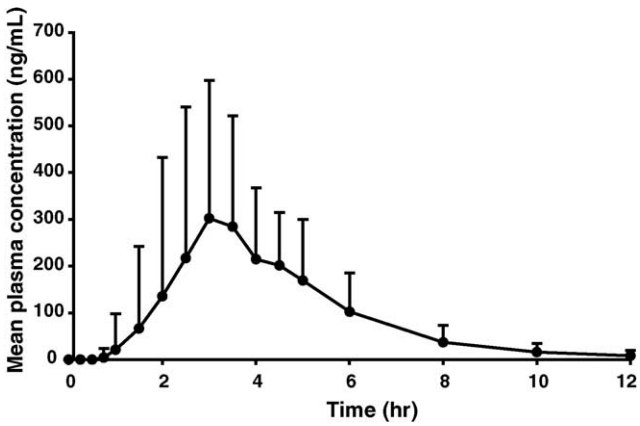

Fig. 3. Mean plasma concentration–time profile of rabeprazole after an oral administration of 20 mg rabeprazole to 18 healthy subjects. Each point represents the mean ± S.D. ($n = 18$).

adjusted 10 with sodium hydroxide solution). The I.S. solution was proved stable at room temperature up to 7.5 h only. To increase the stability of I.S. solution, several pH values of diluent had been evaluated. In addition, several concentrations of buffers, several mixtures of water–acetonitrile or water–methanol had been evaluated to increase the stability of I.S. solution. When the stock solution was prepared in acetonitrile and its working solution (20 μg/mL) by diluting the stock solution with water–acetonitrile (50/50, v/v), the I.S. solution was proved stable at room temperature for more than 24 h and at 4 °C for 14 days. The coefficient of variation was within 3.5%.

3.9. Application to clinical study

The present HPLC method was for the first time employed to determine the pharmacokinetic parameters of rabeprazole in subjects plasma samples of clinical studies. After a single oral dose of 20 mg rabeprazole tablet to 18 healthy subjects, concentration versus time profiles were constructed for up to 12 h for rabeprazole quantitation. Fig. 3 shows the mean concentration–time profile of rabeprazole in 18 healthy subjects following a 20 mg oral dose of rabeprazole under fasting conditions. The maximum rabeprazole mean plasma concentration (C_{max}) was 302 ng/mL, time taken to maximum plasma concentration (t_{max}) was 3.0 h, and half-life ($t_{1/2}$) in the terminal elimination phase was 1.58 h.

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